EXHIBIT H

NAP, a Peptide Derived from the Activity-Dependent Neuroprotective Protein, Modulates Macrophage Function

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ABSTRACT: **NAP is an eight-amino acid neuroprotective peptide NAPVSIPQ; it is the smallest active element derived from the recently cloned activity-dependent neuroprotective protein (ADNP). NAP readily enters the brain from the blood. It will be important to learn whether NAP, in addition to its neuroprotective activity, also might influence immune-mediated inflammation. Here, we report that: (*a*) macrophages express ADNP; (*b*) expression of ADNP in macrophages responds to VIP; and (*c*) NAP downregulates the key inflammatory cytokines tumor necrosis factor (TNF-α), interleukin-16 (IL-16), and IL-12 in macrophages. These findings indicate that ADNP/NAP can play an important role in immune regulation as well as in neuroprotection, which may be mutually related processes.**

KEYWORDS: **vasoactive intestinal polypeptide (VIP); activity-dependent neuroprotective protein (ADNP); macrophages; neuroimmunology; gene expression**

INTRODUCTION

Neurotrophic proteins and neuropeptides have important regulatory functions and are a focus of intensive research in rational drug design.[1,2] Previous

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studies have identified several components by sequential chromatographic methods within the neurotrophic milieu produced by astroglia, activity-dependent neurotrophic factor (ADNF) being one of the most potent.[3] The active peptide fragment of ADNF is ADNF-14 (VLGGGSALLRSIPA)[3]; ADNF-9 (SALLRSIPA), a shorter C-terminal peptide, retains full biological activity.[4] Antibodies to ADNF-14 or to ADNF-9 were used to identify the activity-dependent neuroprotective protein (ADNP), and the cDNA has been cloned from mouse neuroglial cells and human fetal brain.[5,6] Structure/activity screening of several peptides derived from ADNP identified a potent octapeptide, NAP (NAPVSIPQ).[5] NAP has a greater *in vivo* neuroprotective efficacy than ADNF-9.[5,7,8]

The neuroprotective activities of NAP have been studied in a wide variety of systems. NAP induces neuroprotection against the β-amyloid peptide's toxicity involved in the onset of Alzheimer's disease,[5,9,10] oxidative stress,[11] NMDA excitotoxicity,[5] tumor necrosis factor-α (TNF-α) toxicity,[12] transient glucose deprivation,[9] dopamine toxicity, and decreased glutathione.[13] NAP's biological properties related to neuroprotection have been demonstrated in *in vivo* models of closed head injury, fetal alcohol syndrome, and stroke[7,14] and it also been involved in neurodevelopment.[15] NAP is under phase I clinical trials in the United States.

Neuropeptides and neurotrophic proteins perform a broad array of seemingly unrelated functions. Vasoactive intestinal polypeptide (VIP), for example, promotes neuronal survival,[16,17] but is also a potent immunomodulator[18,19] and is under clinical trials. Remarkably, VIP inhibits the acute inflammatory response that follows spinal cord injury[20] and prevents activated microglia-induced neurodegeneration under inflammatory conditions[21] while increasing the synthesis of the NAP-containing protein ADNP in astroglia.[5] Given the breath of NAP's neuroprotective activities and the fact that ADNP is a VIP-responsive gene, we were interested to examine the direct consequences of NAP on the immune system. The present article shows for the first time direct effects of NAP on the macrophage, a cell with a critical role in the initiation and coordination of the immune response. Mindful that VIP acts on activated macrophages as a potent, endogenous anti-inflammatory neuropeptide and that ADNP is a VIP-responsive gene, the current study was performed to investigate whether ADNP mRNA expression can be detected in a mouse macrophage cell line and whether VIP is able to increase the steady-state levels ADNP mRNA.

MATERIAL AND METHODS

Synthetic VIP was purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). NAP was used as before.[22] The mouse macrophage cell line RAW 264.7 was obtained from the American Type Tissue Collection (Rockville, MD). These cells were maintained in RPMI 1640 supplemented

with 25 mM HEPES, 10% (v/v) heat-inactive fetal calf serum (FCS) (Biowhittaker, Wokingham, UK), 10 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (components from Sigma Chemical Co., St. Louis, MO). For mRNA analysis cells, total RNA was extracted and DNAse-treated after TriPure isolation reagent (Roche Diagnostics GmbH, Mannheim, Germany) following manufacturer's instructions. Murine ADNP primers were derived from the published sequence of murine ADNP and reverse transciption polymerase chain reaction (RT-PCR) experimental conditions were previously reported.[23] cDNA was previously titrated to amplify in the linear range. Cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacture's instructions (BD-Pharmingen, San Diego, CA).

RESULTS AND DISCUSSION

RT-PCR of mRNA using the ADNP primers from RAW 264.7 macrophages resulted in single DNA band when analyzed by agarose gel electrophoresis (FIG. 1 A). RT-PCR reactions were also processed with control β-actin house-keeping gene primers. These RT-PCR reactions correspond to the predicted size for PCR amplification using the ADNP primers, and nucleotide sequences of the amplified fragments showed an identical sequence to the mouse ADNP gene.[5] Thus, we report for the first time that ADNP mRNA is expressed in immune system cells, namely, macrophages in a resting state. To further investigate the physiological role of ADNP in this context, we tested whether VIP treatment might influence ADNP gene expression in macrophages. FIGURE 1 A shows that ADNP mRNA levels were increased after 24 h of VIP treatment. The highest increase in ADNP mRNA was produced at nanomolar concentrations of VIP, with a slightly increased level at 10^{-12} M VIP. The effect of VIP was not dose dependent, most probably due to VIP receptor desensitization in macrophages.[24] Thus, ADNP is a VIP-responsive gene in macrophages at concentrations that can be sensed by VIP receptors (VPAC) on immunocompetent cells. VIP production and secretion are elevated after immunological stimuli[25] and, therefore, some of VIP's immunomodulatory properties might be mediated in part by ADNP. To know whether ADNP mRNA levels are modified by immunological stimuli, we incubated RAW 264.7 cells in the presence of increasing concentrations of lipopolysaccharide (LPS) (0.1–10 mg/mL) for 24 h. ADNP gene expression was not altered after LPS treatment (FIG. 1 B). Toll-like receptors (TLRs) are key regulators of innate immunity, sensing and responding to invading microorganisms. LPS is the main ligand of the TLR-4 and TLRs include up to 10 different gene products.

So, at this point, we cannot rule out whether ADNP is regulated by other TLR-ligands. Nevertheless, these data should be taken as qualitative, taking into consideration the limitations of the RT-PCR approach to quantify

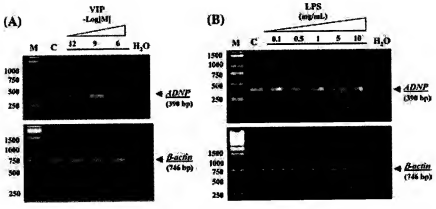

FIGURE 1. ADNP is expressed in macrophages. **(A)** RT-PCR analysis of ADNP in the macrophage-like RAW 264.7 cell line. Arrows indicate the PCR product amplified with specific ADNP primers (390 bp [base pairs]) and β-actin primers (746 bp). **(B)** Effect of LPS treatment on ADNP mRNA levels in RAW 264.7 cells. Results are representative of four independent experiments.

accurately mRNA. In this sense, differential expression of ADNP mRNA is currently being studied in our lab to assess VIP and known TLR-ligands effects by real-time PCR.

Given the breath of NAP's neuroprotective activities and the fact that ADNP is a VIP-responsive gene expressed in macrophages, we were interested to examine direct consequences of NAP treatment on key cytokines involved in the inflammatory response such as TNF-α, interleukin-6 (IL-6), and IL-12. RAW 264.7 cells treated with 0.1 mg/mL LPS in the presence of increasing concentrations of NAP for 24 h showed an inhibition of TNF-α, IL-6, and IL-12 secretion (FIG. 2). Although the mechanism of action involved is not yet known, our results support the role of NAP as a potent immunomodulator. Several questions related to the mechanism of action are under current investigation; we wish to learn whether ADNP could act in a paracrine and/or autocrine fashion under different circumstances. A study on NAP potential new functions can define novel mechanisms that modulate immune responses, and might lead to the development of new therapies for immune-mediated disorders, particularly, for neurodegenerative diseases in which neuronal defense mechanisms and immunomodulation represent innovative approaches.

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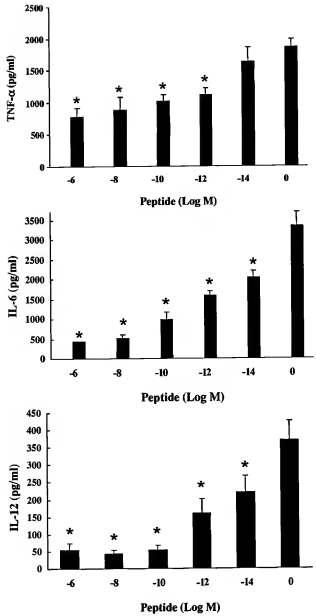

FIGURE 2. Effect of NAP on cytokine release (TNF-α, IL-6, and IL-12) by RAW 264.7 cells. Cytokine levels were determined in supernatants by ELISA after activation of RAW cells with 0.1 mg/mL LPS for 24 h in the presence of different concentrations of NAP. Cytokine basal levels for TNF-α, IL-6, and IL-12 were 485 ± 83 pg/mL, 337 ± 101 pg/mL, and 60 ± 27 pg/mL, respectively. Statistical significance was determined by ANOVA followed by a Student-Newman-Keuls test.

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